

Exhibit 12

Mini Review

Fetal nucleic acids in maternal blood: the promises

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Abstract

Fetal DNA is present at an approximate mean fractional concentration of 10% in the plasma of pregnant women. The detection of paternally-inherited DNA sequences that are absent in the maternal genome, e.g., Y chromosomal sequences for fetal sexing and the *RHD* gene for blood group genotyping, is well established. The recent emergence of single molecule counting technologies, such as digital polymerase chain reaction and massively parallel sequencing has allowed circulating fetal DNA to be used for the non-invasive prenatal diagnosis of fetal chromosomal aneuploidies and monogenic diseases. With large scale clinical validation and further reduction in costs, it is expected that the analysis of circulating fetal DNA will play an increasingly important role in the future practice of prenatal diagnosis.

Keywords: circulating nucleic acids; Down syndrome; next-generation sequencing; noninvasive prenatal diagnosis; plasma DNA; prenatal screening.

Introduction

Prenatal diagnosis is now an established part of modern obstetrics practice. However, the direct analysis of fetal DNA has conventionally relied on the invasive sampling of fetal tissues, e.g., via amniocentesis and chorionic villus sampling, which represents a risk to the fetus. Over the last four decades, much effort has been expended on the development of non-invasive approaches which would obviate such a risk.

In 1997, Lo et al. (1) reported that, during pregnancy, fetal DNA could be detected from the plasma and serum of pregnant women. These investigators showed that in pregnant women carrying male fetuses, Y chromosomal DNA sequences could be detected in their plasma and serum. Subsequent

work using real-time quantitative polymerase chain reaction (PCR) has shown that fetal DNA represents a mean of some 3%–6% of the DNA that is present in maternal plasma (2). Subsequent work using more precise measurement technologies has shown that the actual concentration of circulating fetal DNA in maternal plasma might be double this amount, i.e., at a mean fractional concentration of some 10% (3). It has also been demonstrated that fetal DNA is cleared with a half-life of some 16 min following delivery of the baby (4). This latter observation has thus shown that circulating fetal DNA will not be carried from one pregnancy onto a subsequent one. With the above-mentioned observations, the stage has thus been set for exploring the use of fetal DNA in maternal plasma for non-invasive prenatal diagnosis.

Detection of paternally-inherited sequences

The simplest type of clinical applications using fetal DNA in maternal plasma are those that are based on the detection of sequences that the fetus has inherited from its father and which are absent in the genome of its mother. One example of such sequences involves those that are present on the Y chromosome, which are only present in a male fetus. The presence of such sequences in the pregnant mother's plasma would thus indicate the presence of a male fetus (1, 2). Non-invasive fetal sexing is useful for the prenatal diagnosis of sex-linked diseases (5). For such diseases, the disease-causing gene is present on the X chromosome and thus male fetuses, in whom only one X chromosome is present, are typically more susceptible to such disorders. Another disease in which fetal sexing is useful is congenital adrenal hyperplasia in which the use of dexamethasone treatment to reduce the risk of virilization can be rationalized to cases involving female fetuses (6).

A second example of paternally-inherited DNA sequences that have been detected in maternal plasma is the *RHD* gene (7). This application is useful for determining the RhD blood group status of a fetus carried by a RhD-negative pregnant woman. A number of large scale studies have demonstrated the robustness of this approach of fetal *RHD* genotyping (8, 9).

Because of the robustness of fetal sexing and *RHD* genotyping from maternal plasma, such tests are already in diagnostic use in a number of laboratories.

Detection of fetal chromosomal aneuploidies

The prenatal screening for Down syndrome is perhaps the most sought-after goal for research into non-invasive prenatal

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diagnosis. However, the development of this application is much more challenging to develop than the detection of fetal sex and *RHD* genotype. One reason is that chromosome 21, the chromosome that is involved in Down syndrome, is present in both the fetus and the pregnant mother. Furthermore, one has to devise a method for measuring the dosage of the fetal chromosome 21 in the maternal plasma.

Early work for fetal aneuploidy detection from maternal plasma was typically based on the development of fetal-specific nucleic acid markers. Examples of such markers include those based on the differential DNA methylation between fetal and maternal genomic loci (10, 11) and those based on the detection of mRNA transcribed from genes that are only expressed in the fetus (e.g., in the placenta) (12–14). The first demonstrations that maternal plasma nucleic acids could be used for the direct detection of fetal trisomy 18 and trisomy 21 were achieved in 2006 (11) and 2007 (14), respectively. While these studies have shown in principle that the non-invasive detection of fetal chromosomal aneuploidies is achievable, these approaches are challenging to use in a widespread manner because these strategies are typically based on the measurement of allelic ratios of single nucleotide polymorphisms (11, 14) and each polymorphism is only informative for a proportion of the population.

Recently, Papageorgiou et al. (15) described a method based on the detection of differential DNA methylation between the fetus and mother that is independent of genetic polymorphisms. This method is based on the use of methylated DNA immunoprecipitation followed by real-time PCR analysis of multiple markers. It remains to be seen if the immunoprecipitation step and the performance of the multiple markers can be made robust enough for the approach to be used clinically.

An alternative strategy for achieving the detection of fetal chromosomal aneuploidies is through the use of highly precise methods for quantitation. Thus, when comparing a trisomy 21 with a euploid fetal cell, the proportion of the genome that is contributed by chromosome 21 in the former should be 1.5-times higher than that of the latter. Similarly, the proportional representation of DNA sequences derived from chromosome 21 that is present in the plasma of a pregnant woman carrying a trisomy 21 fetus should be higher than that of a woman carrying a euploid fetus. It has been shown that the amount of increase is half that of the fractional concentration of fetal DNA in maternal plasma (16). One approach for reaching the precision required is by methods that would allow single DNA molecules to be counted (16, 17). Indeed, the larger is the number of DNA molecules that are counted, the higher is the precision.

With the advent of massively parallel sequencing (18), one has a method that would allow millions or even billions of DNA molecules to be counted. During massively parallel sequencing, single DNA molecules are spatially separated and then the molecules are clonally amplified and sequenced, or even sequenced directly without amplification. Two groups have shown that massively parallel sequencing would allow fetal trisomy 21 to be detected non-invasively from maternal plasma (19, 20). This observation has recently been confirmed by a number of subsequent studies (21–24). In particular, the

studies by Chiu et al. (22) and Ehrlich et al. (23) are large scale investigations involving hundreds of samples each.

Early reports suggest that the detection of trisomy 13 and 18 are likely to be more challenging (19–21). However, this issue has recently been solved by the use of particular bioinformatics algorithms (24–26).

Thus, it is expected that the detection of the common fetal chromosomal aneuploidies will be increasingly investigated in the future. Due to the expense associated with massively parallel sequencing, it is likely that this technology will initially be used for pregnant women classified as high risk by conventional screening approaches. However, with the rapid reduction in the costs of massively parallel sequencing, it is possible that this technology would eventually be usable as a first line prenatal test.

Detection of fetal monogenic diseases

With the development of single molecule counting technologies for maternal plasma DNA, new possibilities have also emerged for the non-invasive prenatal diagnosis of fetal monogenic diseases. Thus, one is no longer confined to the detection of DNA sequences that the fetus has inherited from its father but which are absent in its pregnant mother's genome. Instead, one can detect the dosage of a particular mutant gene in the fetus's genome (17, 27). For example, let us consider the scenario of a pregnant woman who is a carrier for an autosomal recessive disorder. The ratio of the mutant and normal gene in her genome is 1:1. Let us consider the scenario that she carries a fetus that is homozygous for the mutant gene. Thus, following the release of the fetal DNA into the maternal plasma, the dosage of the mutant gene should be slightly higher than that for the normal gene. Conversely, if the fetus is homozygous for the normal gene, then the dosage of the normal gene should be slightly higher than that for the mutant gene. Finally, if the fetus is a heterozygous carrier of the disease, similar to its mother, then the dosage of the mutant and normal genes in the mother's plasma should be the same. It has been shown that digital PCR allows one to reach the precision needed for this type of analysis (27). Digital PCR is a technology whereby the DNA template for amplification is adjusted to a concentration whereby most PCR would only contain either a single or no target DNA molecule. When a large number of such amplifications are carried out simultaneously, the number of input template molecules can be measured by counting the number of positive amplification reactions (16, 28). This approach has been successfully realized for the prenatal diagnosis of hemoglobinopathies (27) and hemophilia (29).

With the development of massively parallel sequencing, it has recently been shown that one can deduce a genome-wide genetic map of the fetus using sequencing data from maternal plasma DNA and genetic information from the parents of the fetus (30). Furthermore, with the use of targeted sequencing approaches, in which the power of massively sequencing can be focused on selected genomic regions, one could have a cost-effective way of targeting multiple disease-

causing mutations prevalent in a particular population (31). Discussion concerning the social, practical and ethical implications of these developments has started (32) and should be encouraged in the coming years.

Expert opinion

Since the first discovery of fetal DNA in maternal plasma in 1997, this phenomenon has been rapidly translated into clinical applications. Early diagnostic uses have focused on the detection of paternally-inherited DNA sequences, e.g., for fetal sex determination and fetal *RHD* genotyping. With the development of single molecule counting technologies, such as digital PCR and massively parallel sequencing, such applications have extended to the non-invasive prenatal diagnosis of many monogenic diseases, fetal chromosomal aneuploidies and even for fetal whole genome scanning. It is expected that maternal plasma DNA analysis will play an increasingly important role in the future practice of prenatal diagnosis.

Outlook

With a rapid reduction in the costs of massively parallel sequencing, it is expected that DNA sequencing will move to the center of investigation of the field. For the next 5–10 years, the most active area is expected to be in the use of maternal plasma DNA sequencing for the non-invasive prenatal screening of fetal chromosomal aneuploidies. It is likely that initially the technology will be used predominantly for high risk women, i.e., women who have been screened by other techniques, e.g., nuchal translucency and maternal serum biochemistry. However, with the increasing accessibility of sequencing technologies, it is expected that maternal plasma DNA sequencing will gradually move towards a first line investigation.

Highlights

- Fetal DNA is present at an approximate mean fractional concentration of 10% in maternal plasma.
- The detection of paternally-inherited sequences that are not present in the pregnant woman's genome, e.g., for fetal sex determination and *RHD* blood group genotyping, has been well established.
- Recent development of single DNA molecule counting technologies, such as digital PCR and massively parallel sequencing, allows maternal plasma DNA to be analyzed with an unprecedented degree of precision.
- Massively parallel sequencing of maternal plasma DNA has been shown in a number of studies to allow the sensitive and specific detection of fetal trisomy 21, and more recently also for trisomy 18 and 13.
- Molecular counting technologies allow the prenatal diagnosis of monogenic diseases and even allow the entire fetal genome to be scanned non-invasively.

- Large scale validation of these technologies and further reduction in the costs of these technologies are expected in the coming years.
- Discussion of the social, practical and ethical implications of these developments should be conducted for all stakeholders.

Conflict of interest statement

Author's conflict of interest disclosure: The author holds patents and has filed patent applications in the area of non-invasive prenatal diagnosis. Some of these have been licensed to Sequenom. The author has research funding, consultancy and equity relationships with Sequenom. Patents and relationships with Sequenom played no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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